wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium in the biological sample;

(b) detecting the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and

(c) comparing the signal generated by the calcium indicator molecule at one or more of said time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of said time points in the presence of the candidate agent, and therefrom identifying an agent that alters mitochondrial function.

93. (Amended) The method of any one of claims 1, 21, 64, 80 or 81 wherein subsequent to the step of contacting the biological sample with the source of calcium cations and prior to the step of comparing signals, the biological sample is contacted (i) with at least one compound that uncouples oxidative phosphorylation from ATP production, and (ii) with at least one agent that alters mitochondrial function.

REMARKS

Favorable reconsideration of the instant application in view of the present amendment and the following comments is respectfully requested. Claims 1-96 are under examination in this application; claims 22-42 and 60-63 are canceled by the present amendment without prejudice to the filing of any divisional, continuation or continuation-in-part application, and claims 65-68, 71-74, 78-79 and 93 are amended according to the present amendment solely for purposes of correcting otherwise improper dependencies on canceled claims. Claims 1, 19, 20, 21, 43, 64, 80 and 81 are amended to more particularly point out and distinctly claim subject matter which applicants regard as the invention. Support for amendments of claims 1, 19, 20, 21, 43, 64, 80 and 81 may be found in the instant specification, for example, at page 17, lines 1-16; page 29, line 25 through page 30, line 7; page 31, line 28 through page 32, line 10; page 26, lines

23-27; page 27, lines 14-23; page 61, line 13 through page 66, line 2; page 82, lines 17-28; and in Figures 4, 11 and 12. Attached hereto is a marked-up version of the changes made to the specification and claims by the current Amendment, the first page of which is captioned "**Version with Markings to Show Changes Made.**" (As a courtesy to the Examiner, all currently pending claims appear in the Version with Markings.)

OATH/DECLARATION

In the Action it is alleged that the oath/declaration has not been signed by the second inventor, Amy K. Stout. Applicants are puzzled by this assertion and enclose herewith a photocopy of the original declaration, which is clearly executed by all named inventors, and which was submitted on April 16, 2001, in response to the Notice to File Missing Parts.

DRAWINGS

Applicants thank the Examiner for calling to applicants' attention the need for correction to the labeling in Figures 7-13 and have prepared a complete set of clearly labeled replacement drawings, which are submitted herewith.

AMENDMENT TO THE SPECIFICATION

In response to the requirement for correction asserted in the Action (at paragraph 4), applicants have amended the Brief Description of Drawings to make reference to Figures 13A and 13B, at page 18, lines 7-8 of the specification.

The specification is also objected to for containing embedded hyperlinks. Applicants respectfully submit that the hyperlinks have been inactivated by the present amendment, as set forth above, such that the recited URL addresses will no longer appear as active links. Accordingly, applicants respectfully submit that this objection has been obviated.

CLAIM OBJECTION

The Examiner objects to claims 46 and 48 under 37 CFR 1.75(c), alleging that these claims are in improper dependent form for failing to further limit the subject matter recited in a previous claim from which they depends. More specifically, the Action asserts that

independent claim 43 already recites a calcium ionophore which is a compound that alters intracellular distribution of calcium cation.

Applicants respectfully traverse these grounds for objection and submit that the instant claims are in proper dependent form, in full compliance with 37 CFR 1.75(c). The present invention is directed in pertinent part to a method of identifying an agent that alters mitochondrial function, comprising (a) contacting, in each of a plurality of reaction vessels in a high throughput screening array, (i) a biological sample comprising a cell containing a mitochondrion, cytosol and a calcium indicator molecule, under conditions that permit maintenance of mitochondrial membrane potential, and wherein the calcium indicator molecule is membrane permeable and capable of generating a detectable signal that is proportional to the level of calcium in the cytosol, with (ii) a calcium ionophore, under conditions and for a time sufficient to increase calcium levels within the cell; (b) detecting in each reaction vessel the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and (c) comparing the signal generated by the calcium indicator molecule at one or more of said time points in the absence of the candidate agent, to the signal generated by the calcium indicator molecule at one or more of said time points in the presence of the candidate agent, and therefrom identifying an agent that alters mitochondrial function.

As disclosed in the specification and recited in claim 43, the subject invention method comprises the recited step of contacting (i) a biological sample with (ii) a calcium ionophore. According to certain further embodiments as recited in claims 46 and 48, however, applicants respectfully submit that the biological sample itself (*i.e.*, even *prior to* the recited step of contacting) may comprise a compound that alters intracellular distribution of a calcium cation (claim 46), which compound further may be an ionophore or a membrane permeable compound (claim 48). The specification describes compounds *other than* ionophores that directly or indirectly alter the intracellular distribution of calcium cations (*e.g.*, page 33, line 2 through page 34, line 23; page 58, lines 13-16; page 60, line 8 through page 61, line 22; page 63, line 4 through page 64, line 5), and also contemplates embodiments of the invention method wherein the sample, independent of the recited step of contacting with an ionophore, may optionally

comprise such a compound, or may optionally comprise an ionophore (*e.g.*, page 34, lines 9-23; page 69, line 5 through page 71, line 21). Applicants therefore respectfully submit that the instant claims are in proper dependent form and that this objection has been obviated.

CLAIM REJECTIONS UNDER 35 U.S.C. § 112

The Action rejected claims 1-96 under 35 U.S.C. § 112, second paragraph as being indefinite for allegedly failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. In particular, the Examiner is unclear with regard to whether the recited agent refers to the cytosol, to a previously recited agent or to another agent yet to be added.

Applicants respectfully traverse these grounds for rejection. The present invention is directed generally to a method of identifying an agent that alters mitochondrial function, comprising contacting a biological sample comprising a calcium indicator molecule under conditions that permit maintenance of mitochondrial membrane potential with a source of calcium cations; detecting the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and comparing the signal generated by the calcium indicator molecule at one or more of the time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of the time points in the presence of the candidate agent.

Applicants respectfully submit that the meaning of an agent as recited in the instant claims is clearly disclosed throughout the instant specification, in a manner that would be readily appreciated by one of ordinary skill in the art at the time the instant application was filed. As set forth in the instant specification, for example, at page 45, line 25 through page 47, line 22, a candidate agent for use in the subject invention methods may be any composition of matter that is suspected of altering mitochondrial function, such that an agent identified according to the claimed method is one that alters mitochondrial function. Accordingly, and as described throughout the present application, candidate agents are screened in assays according to the claimed methods, and agents having the ability to alter mitochondrial function are thereby identified (see, *e.g.*, specification at page 18, line 10 through page 21, line 15; page 30, lines 8-

25; page 65, line 1 through page 66, line 2; and in the Examples). Thus, as disclosed in the specification and recited in the claims, the step of comparing signals generated in the absence and presence of a candidate agent relates to the step of detecting the signal generated by the calcium indicator molecule at one or more time points, in the claimed method.

Applicants respectfully submit that the meaning of "agent" is clear, given the instant disclosure, and therefore request that this rejection under 35 U.S.C. § 112, second paragraph be withdrawn.

The Action rejected claims 19-21, 40-42 and 60-62 under 35 U.S.C. § 112, second paragraph, as being indefinite. In particular, the Examiner alleges that the designation (i) and (ii) in step 3 as recited is unclear. More specifically, the Examiner asserts that it cannot be determined whether (i) and (ii) of step 3 refer to a biological sample and source of calcium cations, or whether these subsections refer to substeps.

Applicants respectfully traverse these grounds for rejection. The present invention is directed generally to a method of identifying an agent that alters mitochondrial function (*e.g.*, uncoupling of oxidative phosphorylation (claim 19), inhibition of respiration (claim 20) or calcium uniporter activity (claim 21)), comprising contacting (i) a biological sample comprising a calcium indicator molecule under conditions that permit maintenance of mitochondrial membrane potential with (ii) a source of calcium cations; detecting the signal generated by the calcium indicator molecule at a plurality of time points; and comparing (i) the signal generated by the calcium indicator molecule at one or more of the time points prior to and following at least one of the contacting steps in the absence of a candidate agent, to (ii) the signal generated by the calcium indicator molecule at one or more of the time points prior to and following at least one of the contacting steps in the presence of the candidate agent.

Applicants submit that there is no ambiguity in the meaning of claims 19-21. Applicants are somewhat confused, however, to the reference in the Action to "step 3" of the instant claims where no claim step bears the number three. Specifically, because the Action makes inquiry regarding whether (i) and (ii) refer, respectively, to biological sample and source of calcium cations, applicants believe the Action points to step (a) of the instant claims, where indeed (i) and (ii) refer, respectively, to the recited elements biological sample and source of

calcium cations, and where "(i)" and "(ii)" are included not to indicate substeps of (a) that are themselves action steps, but merely for purposes of clarifying what are the two elements that are the objects of the recited action step of "contacting".

With regard to claims 40-42 and 60-62, applicants submit that the rejection is rendered moot by the present amendment. Accordingly, what is designated by (i) and (ii) in claims 19-21 is clear, given the instant disclosure, and applicants therefore request that this rejection under 35 U.S.C. § 112, second paragraph be withdrawn.

The Examiner rejects claims 63-79 under 35 U.S.C. § 112, second paragraph, as indefinite. In particular, the Examiner alleges that the definition of the term "depleted of cytosol" is unclear with respect to the mitochondria, a component of the cytosol.

Applicants respectfully traverse this rejection. The present invention is directed generally to a method of identifying an agent that alters mitochondrial function, comprising contacting a biological sample comprising a permeabilized cell depleted of cytosol, a mitochondrion and a calcium indicator molecule under conditions that permit maintenance of mitochondrial membrane potential with a source of calcium cations; detecting the signal generated by the calcium indicator molecule at a plurality of time points; and comparing the signal generated by the calcium indicator molecule at one or more of the time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of the time points in the presence of the candidate agent.

Applicants respectfully submit that the term "depleted of cytosol" is clear as recited by the instant claims and as set forth in the specification. For example, at page 41, line 15 through page 42, line 5 the specification describes what is a cell depleted of cytosol, including the disclosure that a cell depleted of cytosol is essentially completely depleted of detectable cytosolic markers such as those described by Fiskum et al. (1980, incorporated by reference, see specification at page 16, lines 3-5) and exemplified by the well known soluble enzyme lactate dehydrogenase (LDH, page 42, lines 3-5). Moreover, applicants respectfully submit that it is well known in the art that "cytosol" refers to the *soluble* portion of cytoplasm (see, *e.g.*, Lehninger, *Biochemistry*-2nd Ed., Worth Publishers, Inc., New York, 1975, page 381, copy

enclosed), while it is similarly established in the art that as organelles, mitochondria are particulate components of cytoplasm, and not soluble components.

Additionally, the specification specifically describes how to prepare cells that are depleted of cytosol, for example, at page 45, lines 10-24, including the express disclosure that pelleted cells depleted of cytosol contain all cellular organelles. Applicants therefore respectfully submit that the instant specification clearly sets forth what is meant by the recitation "depleted of cytosol" in a manner that would be readily appreciated by one of ordinary skill in the art at the time the instant application was filed. Accordingly, Applicants respectfully request that this rejection under 35 U.S.C. § 112, second paragraph, be withdrawn.

CLAIM REJECTION UNDER 35 U.S.C. § 102

Claims 22, 24-26, 28-30, 33, 34, 37, 39, 41, 63 and 81 stand rejected under 35 U.S.C. § 102(b) over Matlib et al. (1998, *J. Biol. Chem. 273(17):*10223-10231). In particular, the Action alleges that Matlib et al. teach time-course assays of the effects of a calcium uniporter inhibitor on calcium uptake by cells *in vitro* and *in vivo,* including calcium uptake by digitonin-permabilized cells or by isolated mitochondria.

Applicants respectfully traverse these grounds for rejection. With regard to claims 22, 24-26, 28-30, 33, 34, 37, 39, 41 and 63, applicants submit that the instant rejections are rendered moot by the cancellation of these claims according to the amendment submitted herewith.

Turning to the subject matter of claim 81, the present invention is directed in pertinent part to a method of identifying an agent that alters mitochondrial function, comprising contacting a biological sample comprising one or more isolated mitochondria and a calcium indicator molecule in a medium, under conditions that permit maintenance of mitochondrial membrane potential with a source of calcium cations; detecting the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and comparing the signal generated by the calcium indicator molecule at one or more of the time points in the absence of a candidate agent, to the signal

generated by the calcium indicator molecule at one or more of the time points in the presence of the candidate agent.

Applicants respectfully submit that, as disclosed in the specification and recited in the instant claim, the present invention comprises in pertinent part a step of detecting a signal generated by a calcium indicator molecule under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity. The invention therefore provides, for the first time, a method for screening candidate agents using isolated mitochondria, wherein the method readily provides the ability to discriminate between several mechanisms by which an agent may alter the calcium concentration in the mitochondrial medium.

In particular, according to the present invention a candidate agent that uncouples or inhibits mitochondrial respiration results in a distinct calcium signal over time (*e.g.*, reversal of calcium uniporter activity, persistence of elevated extramitochondrial calcium concentrations), relative to the signal detected with a candidate agent that inhibits the mitochondrial calcium uniporter (*e.g.*, delayed kinetics of mitochondrial uptake of calcium from the medium), or relative to the signal detected with an agent that has no effect on mitochondrial calcium uptake. For example, and as described in the specification (*e.g.*, at page 17, lines 1-16; page 29, line 25 through page 30, line 7; page 31, line 28 through page 32, line 10; page 61, line 13 through page 66, line 2; page 82, lines 17-28; and in Figure 4), the instant application teaches a person having ordinary skill in the art to select a biological sample comprising mitochondria and a calcium indicator molecule in a medium, under conditions that permit maintenance of mitochondrial membrane potential, and further, to select conditions that permit identification of mitochondrial calcium uniporter activity and mitochondrial uncoupler or respiratory inhibitor activity.

Such conditions include, for instance and as disclosed in the specification at page 64, line 26 through page 66, line 2, monitoring of calcium in the medium of mitochondria following an initial step of contacting the mitochondria with a sequestered pulse of calcium cation, after which a test compound (*e.g.*, candidate agent) is added before a second calcium pulse is applied. A test compound that is a mitochondrial uncoupler or respiratory inhibitor effects release of calcium from the pre-loaded mitochondria, which is detectable as a signal generated by the calcium indicator molecule, while a test compound that is a mitochondrial

calcium uniporter inhibitor would not result in a change in the detectable signal until a subsequent calcium pulse is applied. This is because such a calcium uniporter inhibitor would inhibit mitochondrial uptake (but not release) of calcium cations under the recited conditions, *i.e.*, where mitochondrial membrane potential is maintained. Hence, the instant method (and those of related claims) permits identification of a test compound that is a mitochondrial uncoupler or respiratory inhibitor.

Additionally, when the test compound has been determined not to be a mitochondrial uncoupler or respiratory inhibitor by the above criteria, and when a subsequent calcium pulse is applied, a test compound that alters mitochondrial calcium uniporter activity can be identified as one that effects an altered profile of mitochondrial calcium uptake (and a concomitant change in the detectable signal generated by the calcium indicator molecule), such as a mitochondrial calcium uniporter activity inhibitor, which would impair mitochondrial uptake of calcium and thereby prolong the period during which a calcium signal is detected in the extramitochondrial medium. Therefore, the instant method permits identification of mitochondrial calcium uniporter activity and of a test compound that alters such activity. Additional examples of the subject invention methods (*i.e.*, including but not limited to the method according to the instant claim 81, which relates to use of a sample comprising isolated mitochondria) are described in greater detail in the specification, at pages 82-91 and in Figure 10 (showing identification of mitochondrial uncoupler activity using the uncoupler compound FCCP) and Figure 12 (showing identification of mitochondrial calcium uniporter activity using Ru-360, and of mitochondrial uncoupler activity using FCCP).

By contrast, Matlib et al. fail, *inter alia*, to disclose conditions that permit specific identification of mitochondrial calcium uniporter activity, *and* of mitochondrial uncoupler or respiratory inhibitor activity, because any assays described by Matlib et al. fail to contemplate a method capable of making such distinctions. Specifically, Matlib et al. fail to contemplate selection of assay reagents and conditions that permit differential profiling over time of the detectable calcium concentration in a sample, where such profiling permits determination of whether a candidate agent alters mitochondrial calcium uniporter activity and also permits determination of whether a candidate agent is a mitochondrial uncoupler or respiratory inhibitor. Applicants submit that given the teachings of Matlib et al., a person having ordinary skill in the

art would not be able to discern a test compound that is a mitochondrial uncoupler from one that is a calcium uniporter inhibitor. Applicants therefore submit that Matlib et al. fail to disclose the claimed invention, where it is well settled that a claim is anticipated "only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference" (*Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987)), and that for a reference to anticipate a claimed invention, "[t]he identical invention must be shown in as complete detail as it is contained in the . . . claim." (*Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989)). Moreover, the elements must be arranged as required by the claim. *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed. Cir. 1990). See also MPEP 2131.

Matlib et al. merely describe measurements of calcium cation uptake and release by isolated mitochondria in the absence or presence of a ruthenium red derivative, Ru-360, which is a known calcium uniporter inhibitor, using arsenazo III. Nowhere do Matlib et al. teach or even remotely suggest use of the assay described therein for screening candidate agents using isolated mitochondria, nor for detecting a signal from isolated mitochondria using a calcium indicator molecule at a plurality of time points, nor determination of conditions suitable for the identification of mitochondrial calcium uniporter activity *per se, i.e.*, as distinguished from mitochondrial uncoupler or respiratory inhibitor activity. The only time-course studies disclosed by Matlib et al. involve whole-cell (Figs. 6-9) and patch-clamp (Fig. 6) techniques, which applicants submit neither employ isolated mitochondria nor provide conditions that permit identification of mitochondrial calcium uniporter and uncoupler or respiratory inhibitor activities. Furthermore, according to certain experiments described by Matlib et al., respiratory inhibitors such as rotenone or oligomycin are present, which applicants submit would preclude the recited condition of the instant claim that the sample be contacted with a source of calcium cations under conditions that permit maintenance of mitochondrial membrane potential.

Accordingly, applicants submit that Matlib et al. fail to disclose each and every limitation of the instant claim, and that the rejection has therefore been overcome. Reconsideration of this rejection and its withdrawal in view of the present amendment are respectfully requested.

CLAIM REJECTIONS UNDER 35 U.S.C. § 103

A. Claims 23, 27, 31, 38, 40, 60, 61 and 62 stand rejected under 35 U.S.C. 103(a) as unpatentable over Matlib et al. (1998, *J. Biol. Chem. 273(17):*10223-10231), in view of McCormack et al. (1989, *Biochimica et Biophysica Acta 973*:420-427). In particular, the Action (paragraph 9) alleges that Matlib et al. teach an assay measuring the affect of Ru360 on Ca^{2+} uptake into mitochondria and that McCormack et al. teach the use of a calcium ionophore, the use of a repeated calcium pulse, and the use of a mitochondrial uncoupler. The Action then alleges that it would have been *prima facie* obvious for one of ordinary skill in the art at the time the instant application was filed to combine these assay steps to arrive at the claimed invention.

Applicants respectfully submit that these rejections have been rendered moot by the present amendment, according to which the instant claims have been canceled without prejudice. Accordingly applicants request that these rejections be withdrawn.

B. Claims 1, 3-9, 12, 13, 16-21, 54, 55, 58, 59, 64-66, 68, 69, 71, 74, 75, 78-80, 82, 83, 85, 86, 89 and 90 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Matlib et al. (1998, *J. Biol. Chem. 273(17):*10223-10231), in view of Marban et al. (U.S. Patent No. 6,183,948). In particular, the Action (paragraph 10) alleges that Matlib et al. (discussed *supra*) do not teach a high throughput assay but that high throughput assays are provided by Marban et al., such that it would have been *prima facie* obvious for a person having ordinary skill in the art to adapt high throughput embodiments to the assay of Matlib et al. to arrive at the claimed invention.

Applicants respectfully traverse these grounds for rejection. The present invention is directed in pertinent part to a method of identifying an agent that alters mitochondrial function, comprising contacting a biological sample comprising a calcium indicator molecule under conditions that permit maintenance of mitochondrial membrane potential with a source of calcium cations; detecting the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor

activity; and comparing the signal generated by the calcium indicator molecule at one or more of the time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of the time points in the presence of the candidate agent.

Applicants respectfully submit that Matlib et al., alone or in combination with Marban et al., fail to teach or suggest the limitations of the present invention. Matlib et al. fail in any way to suggest use of the assays described therein as screening assays and certainly not as high throughput screening assays, particularly insofar as certain of the experiments disclosed by Matlib et al. relate to perfusion and patch-clamp techniques that are not readily amenable to high throughput screening. As also noted above, Matlib et al. fail to contemplate a method comprising, *inter alia*, the recited step of detecting a signal generated by a calcium indicator molecule under conditions (including the choice of calcium indicator molecule) that have been selected to permit identification of mitochondrial calcium uniporter activity and of mitochondrial uncoupler or respiratory inhibitor activity. In particular, Matlib et al. fail to teach or suggest the desirability of such conditions, nor do Matlib et al. articulate any recognition of the desirability of an assay design that would permit identification of a mechanism involving mitochondrial calcium uniporter activity and mitochondrial uncoupler or respiratory activity.

The disclosure of Marban et al. relates generally to assays of mitochondrial function, including screening assays, but applicants submit that given the teachings of Matlib et al. and of Marban et al., a person having ordinary skill in the art at the time of filing the instant application would not have been motivated specifically to arrive at the presently claimed invention with any reasonable expectation of success.

Marban et al. merely describe screening assays that comprise monitoring changes in endogenous cellular fluorescence that result from alterations in a mitochondrial redox state within cells. Marban et al. fail, however, to teach or suggest a method comprising the use of a calcium indicator molecule that is capable of generating a detectable signal that is proportional to the level of calcium in a cell, in cytosol or in a mitochondrial medium as provided by the present invention, nor does the Action anywhere point to such a suggestion in Marban et al. The assays of Marban et al. are not specific assays of intracellular calcium levels, and certainly cannot be specific assays of mitochondrial calcium transport activity such as a mitochondrial calcium uniporter activity, because the endogenous fluorescent redox indicator disclosed by Marban et al.

may generate a signal indicative of altered redox conditions in response to any of a number of non-specific stimuli that are wholly independent of the mitochondrial calcium uniporter. Applicants respectfully submit that neither do Marban et al. suggest the desirability of combining the teachings therein with Matlib et al. or with any other reference known to the art, to arrive at the presently claimed invention, especially where these references or any other disclosures known to the art fail to provide assay conditions that permit distinguishing mitochondrial uncouplers or respiratory inhibitors from mitochondrial calcium uniporter activity inhibitors. The combination of Marban et al. and Matlib et al. is silent with regard to assay conditions (including, *e.g.,* the choice of calcium indicator molecule) that permit identification of mitochondrial calcium uniporter activity and of mitochondrial uncoupler or respiratory inhibitor activity. In other words, and as discussed above, absent the teachings of the present application a person having ordinary skill in the art would not, with any reasonable expectation of success, have been able to select a calcium indicator molecule and conditions for distinguishing between (i) a candidate agent that alters mitochondrial or extramitochondrial calcium levels by altering mitochondrial calcium uniporter activity, and (ii) a candidate agent that alters mitochondrial or extramitochondrial calcium levels by having mitochondrial uncoupler or respiratory inhibitor activity. Applicants submit that the Action employs impermissible hindsight in view of the instant application to assert otherwise, especially where the Action fails specifically to point to any suggestion in either of the cited references to combine elements known to the art, to arrive at the claimed invention.

Furthermore, applicants respectfully submit that the mere fact that the teachings of the prior art *can* be combined or modified, or that a person having ordinary skill in the art is *capable* of combining or modifying the teachings of the prior art, does not make the resultant combination *prima facie* obvious, as the prior art must also suggest the desirability of the combination (*see, e.g., In re Mills*, 16 USPQ2d 1430, Fed. Cir., 1990; *In re Fritch*, 23 USPQ2d 1780, Fed. Cir., 1992). In addition, the prior art references when combined must teach or suggest all the claim limitations (*see, e.g., In re Royka*, 180 USPQ 580, CCPA, 1974). Thus, applicants respectfully submit that the Office Action has not set forth a *prima facie* case of obviousness, where the cited references fail to teach every limitation of the instant invention and fail to

provide motivation for a person having ordinary skill in the art to modify or combine the prior art teachings to arrive at the claimed invention with a reasonable expectation of success.

Therefore, applicants submit that it would not have been *prima facie* obvious to apply the high throughput methods of Marban et al. to the assays described by Matlib et al in order to screen a plurality of samples for their effect on mitochondrial function. Accordingly, applicants respectfully submit that the instant claims satisfy the requirements of 35 U.S.C. § 103(a) and request that these rejections be withdrawn.

C. Claims 2, 10, 43-52, 54, 55, 58, 59, 67, 70, 72, 84, 87, 93 and 96 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Matlib et al. (1998, *J. Biol. Chem. 273(17)*:10223-10231), in view of Marban et al. (U.S. Patent No. 6,183,948), in further view of McCormack et al. (1989, *Biochimica et Biophysica Acta 973*:420-427). In particular, the Action (paragraph 11) concedes that Matlib et al. (discussed *supra*) do not teach further adding an ionophore, but the Action alleges that it would have been *prima facie* obvious for a person having ordinary skill in the art, based on the teachings of McCormack, to add ionophore and to permeabilize mitochondria in order to increase calcium cation stabilization. The Action asserts further that it would have been obvious to arrive at the claimed invention to achieve an accurate measure of calcium cation concentration using ionomycin and FCCP, according to the teachings of McCormack et al.

Applicants respectfully traverse these grounds for rejection. The present invention is directed in pertinent part to a method of identifying an agent that alters mitochondrial function, comprising contacting a biological sample comprising a calcium indicator molecule under conditions that permit maintenance of mitochondrial membrane potential with a source of calcium cations; detecting the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and comparing the signal generated by the calcium indicator molecule at one or more of the time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of the time points in the presence of the candidate agent.

Applicants respectfully submit that Matlib et al., Marban et al. and McCormack et al., alone or in any combination, fail to teach or suggest the methods encompassed by the instant claims. Matlib et al. and Marban et al. are discussed above. Briefly, Marban et al. fail to provide anything more than a generic suggestion that a mitochondrial function may be assayed by endogenous fluorescence (*i.e.*, not using a calcium indicator molecule according to the instant invention) in a high throughput screening format. As also noted above, Marban et al. disclose only a highly *non-specific* assay using an endogenous intracellular redox indicator that may generate a signal in response to any number of stimuli however unrelated to mitochondrial calcium uniporter activity, or even to any type of mitochondrial calcium transport. Marban et al. or Matlib et al., alone or in combination, therefore fail to teach or suggest a screening assay or a high throughput screening assay in a method of identifying an agent that alters mitochondrial function, which assay comprises detecting a calcium indicator molecule under conditions that permit identifying mitochondrial calcium uniporter activity and mitochondrial uncoupler or respiratory inhibitor activity. Applicants submit further that McCormack et al. fail to remedy the deficiencies of Matlib et al. and Marban et al. because, *inter alia*, McCormack et al. only describe assays that are *not* conducted under conditions that permit maintenance of mitochondrial membrane potential; the teachings of McCormack et al. are also limited to assays using isolated mitochondria. Moreover, McCormack et al. fail to teach or suggest a screening method wherein detection of a signal generated by a calcium indicator molecule is performed under conditions that permit identification of mitochondrial calcium uniporter activity and mitochondrial uncoupler or respiratory inhibitor activity. Thus, like Matlib et al. and Marban et al., McCormack et al. fail to anticipate the instant invention where no assay described therein would have motivated an ordinarily skilled artisan to arrive at the subject invention assay method, which permits such mechanistic characterization (*i.e.*, uniporter inhibitor *vs.* uncoupler/ respiratory inhibitor) of a candidate agent based on the detected signal.

McCormack et al. describe fluorescence experiments using isolated rat heart mitochondria under conditions in which the mitochondria are not permitted to maintain mitochondrial membrane potential. McCormack et al. employ a calcium-sensitive fluorescent indicator molecule and expose the mitochondria to ionomycin to equilibrate the mitochondrial matrix and extramitochondrial Ca^{2+} pools. As described by McCormack et al., ionomycin

treatment selectively permeabilizes mitochondria to calcium cations, thereby overriding any endogenously regulated mitochondrial calcium transport activity such as mitochondrial calcium uniporter activity. As well, McCormack et al. describe the use of a mitochondrial uncoupler and thereby cause dissipation of mitochondrial membrane potential. By way of contrast, according to the present invention the recited step of contacting the sample with a source of calcium cations takes place under conditions that permit calcium uniporter activity to take place, thereby permitting agents that alter calcium uniporter activity to be identified. Thus, while certain embodiments of the present invention relate to the use of permeabilized *cells*, the claimed methods do not contemplate *mitochondria* that are artificially rendered calcium-permeable according to the disclosure of McCormack et al.

Applicants therefore submit that if anything, the disclosure of McCormack et al. teaches away from the presently claimed invention because the cited reference teaches that the presence of ionomycin precludes detection of mitochondrial calcium uniporter activity, while the instant claims are directed to a method comprising the recited step of detecting under conditions that permit identification of mitochondrial calcium uniporter activity. Therefore, applicants submit that it would not have been *prima facie* obvious to combine the teachings of McCormack et al., Marban et al. and Matlib et al in order to screen a plurality of samples for their effect on mitochondrial function. Accordingly, applicants respectfully submit that the instant claims satisfy the requirements of 35 U.S.C. § 103(a) and request that these rejections be withdrawn.

D. Claims 94 and 95 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Matlib et al. (1998, *J. Biol. Chem. 273(17):*10223-10231), in view of Marban et al. (U.S. Patent No. 6,183,948) and McCormack et al. (1989, *Biochemica et Biophysica Acta 973*: 420-427), in further view of Bernardi et al. (1993, *J. Biol. Chem. 268*: 1005-1010). In particular, the Action (paragraph 12) alleges that Bernardi et al. teach the effect of cyclosporin on mitochondrial permeability transition (MTP), and that a person having ordinary skill in the art would have been motivated to apply the teaching of Bernardi et al. (cyclosporin A) to the assay of Matlib et al. to examine the MPT inhibitory effect on Ca^{2+} uptake.

Applicants respectfully traverse this rejection. The present invention is directed in pertinent part to a method of identifying an agent that alters mitochondrial function, comprising

contacting a biological sample comprising a calcium indicator molecule under conditions that permit maintenance of mitochondrial membrane potential with a source of calcium cations; detecting the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; comparing the signal generated by the calcium indicator molecule at one or more of the time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of the time points in the presence of the candidate agent; and, subsequent to the step of contacting the biological sample with the source of calcium cations and prior to the step of comparing signals, contacting the biological sample with (i) at least one compound that uncouples oxidative phosphorylation from ATP production, and (ii) at least one agent that alters mitochondrial function, wherein the agent that alters mitochondrial function is cyclosporin A, rotenone, oligomycin, succinate or Bcl-2.

For reasons set forth above, applicants submit that Matlib et al., Marban et al. and/or McCormack et al., alone or in any combination, fail to teach or suggest the method encompassed by claim 93, from which the instant claims depend. Applicants submit further that the addition of Bernardi et al. fails to render obvious these or any other claims of the instant application. Bernardi et al. merely disclose the effects of calcium cations and uncouplers on permeability transition in isolated rat liver mitochondria, where Bernardi et al. only describe assays in which mitochondrial membrane potential or mitochondrial matrix pH are measured. Specifically, Bernardi et al. demonstrate that cyclosporin A can inhibit mitochondrial permeability transition (MPT) under certain conditions. The disclosure of Bernardi et al. is therefore merely cumulative with disclosure in the present application of compounds, including cyclosporin, that alter mitochondrial function and which may optionally be included in certain embodiments of the invention (*e.g.*, specification at page 21, line 13). Even combined with Matlib et al., Marban et al. and/or McCormack et al., however, Bernardi et al. fail to contemplate the subject invention assay method, in which a single set of assay conditions permits an ordinarily skilled artisan to distinguish between a candidate agent that alters mitochondrial calcium uniporter activity and a candidate agent that has mitochondrial uncoupler or respiratory inhibitor activity, by detecting the signal generated by a calcium indicator molecule.

Accordingly, applicants submit that even in view of the other references cited in the Action, the mere disclosure by Bernardi et al. that cyclosporin A influences MPT, where an association between calcium cations and MPT was long known (*e.g.*., specification at page 3, line 14 through page 4, line 19; page 23, lines 10-30), fails to anticipate the instant claims. Applicants therefore traverse the assertion in the Action and submit that no *prima facie* case of obviousness has been established, where the Examiner has provided neither evidence nor reasoning to support the allegation that the cited references would have motivated the ordinarily skilled artisan to arrive at the instant invention absent the disclosure of the present application. In particular, and for reasons discussed in greater detail above, simply combining cyclosporin A in the assay of Matlib et al. would fall far short of any suggestion of the present invention, where Matlib et al. fail to contemplate a method comprising, *inter alia*, the recited step of detecting a signal generated by a calcium indicator molecule under conditions (including the choice of calcium indicator molecule) that have been selected to permit identification of mitochondrial calcium uniporter activity and of mitochondrial uncoupler or respiratory inhibitor activity.

Applicants therefore respectfully submit that the instant claims satisfy the requirements of 35 U.S.C. § 103(a) and request that these rejections be withdrawn.

E. Claims 11, 14, 32, 35, 88 and 91 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Matlib et al. (1998, *J. Biol. Chem. 273(17):*10223-10231), in view of Marban et al. (U.S. Patent No. 6,183,948), in further view of Murphy et al. (1996, *PNAS 93*:9893-9898). In particular, the Action (paragraph 13) alleges that it would have been *prima facie* obvious for a person having ordinary skill in the art to apply the teaching of Murphy et al., that Bcl-2 enhances mitochondrial sequestration of calcium without respiratory impairment, to the assay of Matlib et al., in order to study the calcium uptake effect on cell death.

Applicants respectfully traverse these grounds for rejection. With regard to claims 32 and 35, applicants submit that the instant rejections are rendered moot by the cancellation of these claims according to the amendment submitted herewith.

Turning to the subject matter of claims 11, 14, 88 and 91, the present invention is directed in pertinent part to a method of identifying an agent that alters mitochondrial function, comprising contacting in a high throughput array a biological sample and a calcium indicator

molecule in a medium, under conditions that permit maintenance of mitochondrial membrane potential with a source of calcium cations; detecting the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and comparing the signal generated by the calcium indicator molecule at one or more of the time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of the time points in the presence of the candidate agent. According to claims 11 and 88, the sample comprises at least one polypeptide that is a Bcl-2 family member; according to claims 14 and 91, the sample is derived from a cell that expresses a transfected gene encoding a polypeptide that regulates cytosolic calcium.

As also discussed in greater detail above, the combination of Matlib et al. and Marban et al. fails to teach or suggest the present invention. Applicants submit that Murphy et al., alone or in combination with Matlib et al. and/or Marban et al., fail to anticipate the subject matter of the instant claims. Murphy et al. disclose that Bcl-2 expressing cells, including cells expressing a Bcl-2-encoding transgene, exhibit enhanced ability to sequester calcium in mitochondria. However, Murphy et al. fail to contemplate, *inter alia*, use of the method described therein for screening candidate agents in a method of identifying an agent that alters mitochondrial function, nor do Murphy et al. teach or suggest a high throughput screening array. Applicants therefore further respectfully submit that the disclosure of Murphy et al. includes no teaching or suggestion that any assay described therein is desirably designed to include conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity, and in particular, that Murphy et al. fail to provide for determining whether an agent that could be screened according to the teachings therein might influence either of these activities. As these deficiencies of Murphy et al. are not remedied by Matlib et al. and Marban et al. for reasons set forth above, and in particular where the Action fails to point to any suggestion in any of the cited references to combine its teachings with those found in any other of the cited references or known to the art to achieve the instant invention, applicants submit that the Action has failed to establish a case of *prima facie* obviousness.

Applicants therefore respectfully submit that the instant claims comply with the requirements of 35 U.S.C. § 103(a) and request that these rejections be withdrawn.

F. Claims 53, 56, 73 and 76 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Matlib et al. (1998, *J. Biol. Chem. 273(17):*10223-10231), in view of Marban et al. (U.S. Patent No. 6,183,948) and McCormack et al. (1989, *Biochimica et Biopysica Acta 973*:420-427), in further view of Murphy et al. (1996, *PNAS 93*:9893-9898). In particular, the Action (paragraph 14) alleges that a person having ordinary skill in the art would have been motivated to apply teachings of Murphy et al., regarding Bcl-2-enhanced sequestration of mitochondrial calcium without respiratory impairment, to the assay of Matlib et al. in order to study the Ca^{2+} uptake effect on cell death.

Applicants respectfully traverse this rejection. The present invention is directed in pertinent part to a method of identifying an agent that alters mitochondrial function, comprising contacting in a high throughput array a biological sample (either a cell according to claims 53 and 56, or a permeabilized cell depleted of cytosol according to claims 73 and 76) and a calcium indicator molecule in a medium, under conditions that permit maintenance of mitochondrial membrane potential with a source of calcium cations; detecting the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and comparing the signal generated by the calcium indicator molecule at one or more of the time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of the time points in the presence of the candidate agent. According to claims 53 and 73, the sample comprises at least one polypeptide that is a Bcl-2 family member; according to claims 56 and 76, the sample is derived from a cell that expresses a transfected gene encoding a polypeptide that regulates cytosolic calcium.

Applicants are somewhat puzzled by the citation to McCormack et al. in the instant rejections since, for reasons given above, McCormack et al. describe only assays using isolated mitochondria under conditions (*e.g.*, ionomycin/ uncoupler) wherein maintenance of

mitochondrial membrane potential is not permitted. Accordingly, applicants respectfully submit that the disclosure of McCormack et al. is inapposite to the instant claims.

As also discussed in greater detail above, applicants submit that the mere disclosure in Marban et al. of a high throughput screening assay applicable generally to mitochondrial function, without more, makes this reference inapposite to the instant claims as well, even in combination with the other cited references. Where Marban et al. fail in any way to contemplate, for instance, a screening assay for an agent that alters mitochondrial function wherein mitochondrial calcium uniporter activity can be discerned from mitochondrial uncoupler or respiratory inhibitor activity, and where Marban et al. further fail to teach or suggest a calcium indicator molecule that is selected according to the teachings of the present application, applicants submit that no *prima facie* case of obviousness has been established because no motivation to combine the cited references has been shown in the Action.

Applicants therefore also respectfully submit that the subject matter of the instant claims can be readily distinguished over any teaching or suggestion found in any one or more of Matlib et al., Marban et al., McCormack et al. and Murphy et al. Specifically, and as discussed above, the disclosure of Murphy et al. is limited to a description of the Bcl-2-mediated enhancement of mitochondrial calcium capacity without impairment of mitochondrial respiratory activity. The Action, however, fails to point to any suggestion in Murphy et al. or in any of the other cited references to combine, with a reasonable expectation of success, a Bcl-2 expressing cell or a transfected cell according to Murphy et al. in a screening assay as recited by the instant claims. On this point, applicants have already noted several times herein that the collective prior art falls short of providing the requisite motivation, absent the teachings of the instant application, to achieve the present invention. Specifically, the combination of, *inter alia*, high throughput screening array format, maintenance of mitochondrial membrane potential, selection of calcium indicator molecule and ability to identify (*i.e.*, distinguish) mitochondrial calcium uniporter activity and mitochondrial uncoupler or respiratory inhibitor activity, is simply nowhere suggested in the prior art.

Accordingly, applicants submit that the instant claims comply with the requirements of 35 U.S.C. §103 and request that these rejections be withdrawn.

All of the claims remaining in the application are now clearly allowable. Favorable consideration and a Notice of Allowance are earnestly solicited.

Respectfully submitted,

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Enclosure:
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VERSION WITH MARKINGS TO SHOW CHANGES MADEIn the Specification:

Please amend the paragraph at page 18, lines 7-8 to read as follows:

Figure 13 shows the impact of an MPT effector, diamide, on results obtained using the assay of the invention in the absence (Fig. 13A) and presence (Fig. 13B) of CsA.

Please amend the paragraph at page 31, lines 8-27 to read as follows:

Depending on the assay, a Fluorometric Imaging Plate Reader (FLIPR™) instrument (Molecular Devices, Sunnyvale, CA) is often the instrument of choice for fluorescence-based assays of the invention. The FLIPR™ system ~~(see http://www.moleculardevices.com/pages/flipr.html)~~ (Molecular Devices, Sunnyvale, CA) has the following desirable features: (i) It uses a combination of a water-cooled, argon-ion laser illumination and cooled CCD camera as an integrating detector that accumulates detectable signal over the period of time in which it is exposed to the image and, as a result, its signal-to-noise characteristics are generally superior to those of conventional imaging optics; (ii) it also makes use of a proprietary cell-layer isolation optics that allow signal discrimination on a cell monolayer, thus reducing undesirable extracellular background fluorescence; (iii) it provides data in real-time, and can also provide kinetic data (*i.e.*, readings at a plurality of timepoints); (iv) it has the ability to simultaneously excite fluorophores in, and read emissions from, all 96 wells of a 96-well microplate; (v) it provides for precise control of temperature and humidity of samples during analysis; (vi) it includes an integrated state-of-the-art 96-well pipettor, which uses dispensable tips to eliminate carryover between experiments, and that can be used to aspirate, dispense and mix precise volumes of fluids from microplates; and, (vii) in the case of the FLIPR^{384} instrument, it can be adapted to run sample assays in a robotic or semi-robotic fashion, thus providing for rapid HTS analysis of large numbers of samples (*e.g.*, up to about a hundred 96-well microplates per day).

Please amend the paragraph at page 70, line 20 through page 71, line 21 to read as follows:

A variety of apoptogens are known to those familiar with the art and may include by way of illustration herbimycin A (Mancini et al., *J. Cell. Biol. 138*:449-469, 1997); paraquat (Costantini et al., *Toxicology 99*:1-2, 1995); ethylene glycols (~~http://~~www.ulaval.ca/vrr/rech/Proj/532866.html); protein kinase inhibitors such as, *e.g.*: staurosporine, calphostin C, caffeic acid phenethyl ester, chelerythrine chloride, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine, N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-5-isoquinolinesulfonamide, KN-93, genistein, quercitin and d-*erythro*-sphingosine derivatives; ultraviolet radiation; ionophores such as, *e.g.*, ionomycin, valinomycin and other ionophores known in the art; MAP kinase inducers such as, *e.g.*, anisomycin and anandamine; cell cycle blockers such as, *e.g.* aphidicolin, colcemid, 5-fluorouracil and homoharringtonine; acetylcholineesterase inhibitors such as, *e.g.*, berberine; anti- estrogens such as, *e.g.*, tamoxifen; pro-oxidants such as, *e.g.*, tert-butyl peroxide and hydrogen peroxide; free radicals such as, *e.g.*, nitrous oxide; inorganic metal ions, such as, *e.g.*, cadmium; DNA synthesis inhibitors such as, *e.g.*, actinomycin D, bleomycin sulfate, hydroxyurea, methotrexate, mitomycin C, camptothecin, daunorubicin and DNA intercalators such as, *e.g.*, doxorubicin; protein synthesis inhibitors such as, *e.g.*, cycloheximide, puromycin, and rapamycin; agents that effect microtubule formation or stability such as, *e.g.*: vinblastine, vincristine, colchicine, 4-hydroxyphenylretinamide and paclitaxel; gangliosides such as GD3 (Scorrano et al., *J. Biol. Chem. 274*:22581-22585, 1999); agents that may be contacted with cells having appropriate receptors including, by way of example and not limitation, tumor necrosis factor (TNF), FasL, glutamate, NMDA (the preceding are contacted with cells having receptors that mediate the uptake of the indicated agent), corticosterone [with cells having mineral corticoid or glucocorticoid receptor(s)]; agents that are withdrawn from the culture media of cells after some period of time such as, by way of non-limiting example, the withdrawal of IL-2 from lymphocytes; and agents that can be contacted with isolated mitochondria or permeabilized cells including, by way of example and not limitation, calcium and inorganic phosphate, (Kroemer et al., *Ann. Rev. Physiol. 60*:619-642, 1998) and members of the Bax/Bcl-2 family of proteins (Jurgenmeier et al., *Proc. Natl. Acad. Sci. U.S.A. 95*:4997-5002, 1998). Such agents are prepared according to methods known in the art or are commercially available from companies such as, for example, Calbiochem (San Diego, CA) and Sigma Chemical Company (St. Louis, MO).

Please amend the paragraph at page 73, lines 9-27 to read as follows:

Depending on the assay, a Fluorometric Imaging Plate Reader (FLIPR^{TM}) instrument (Molecular Devices, Sunnyvale, CA) is often the instrument of choice for the assays of the invention. The FLIPR^{TM} (Molecular Devices, Sunnyvale, CA) has the following desirable features (see ~~http://~~www.moleculardevices.com/pages/flipr.html): it uses a combination of a water-cooled, argon-ion laser illumination and cooled CCD camera as an integrating detector that accumulates signal over the period of time in which it is exposed to the image and, as a result, its signal-to-noise characteristics are generally superior to those of conventional imaging optics; it also makes use of a proprietary cell-layer isolation optics that allow signal discrimination on a cell monolayer, thus reducing undesirable extracellular background fluorescence; it provides data in real-time, and can also provide kinetic data (*i.e.*, readings at a multitude of timepoints); it has the ability to simultaneously stimulate and read all 96 wells of a 96-well microplate; it provides for precise control of temperature and humidity of samples during analysis; it includes an integrated state-of-the-art 96-well pipettor, which uses dispensible tips to eliminate carryover between experiments, that can be used to aspirate, dispense and mix precise volumes of fluids from microplates; and, in the case of the FLIPR^{384} instrument, it can be adapted to run sample assays in a robotic or semi-robotic fashion, thus providing for analysis of large numbers of samples in shortest amount of time (*e.g.*, up to about a hundred 96-well microplates per day).

In the Claims:

Claims 22-42 and 60-63 are canceled without prejudice.

Claims 1, 19, 20, 21, 43, 64, 65-68, 71-74, 78-79, 80, 81 and 93 are amended to read as follows (as a courtesy to the Examiner, all currently pending claims appear here):

1. (Amended) A method of identifying an agent that alters mitochondrial function, comprising:

(a) contacting, in each of a plurality of reaction vessels in a high throughput screening array,

(i) a biological sample comprising a cell containing cytosol, a mitochondrion and a calcium indicator molecule, under conditions that permit maintenance of mitochondrial membrane potential, with

(ii) a source of calcium cations,

wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium in the cytosol;

(b) detecting in each reaction vessel the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and

(c) comparing the signal generated by the calcium indicator molecule at one or more of said time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of said time points in the presence of the candidate agent, and therefrom identifying an agent that alters mitochondrial function.

2. The method of claim 1 wherein the step of contacting is repeated at least once.

3. The method of claim 1 wherein the sample contains at least one compound that alters intracellular distribution of a calcium cation.

4. The method of claim 3 wherein the compound that alters intracellular calcium cation distribution is selected from the group consisting of thapsigargin and Ru360.

5. The method of claim 3 wherein the compound that alters intracellular calcium cation distribution is selected from the group consisting of a calcium ionophore and a membrane permeable compound that alters intracellular calcium distribution.

6. The method of claim 3 wherein the sample contains at least one compound that uncouples oxidative phosphorylation from ATP production.

7. The method of claim 1 wherein the candidate agent is membrane permeable.

8. The method of claim 1 wherein the calcium indicator molecule is membrane permeable.

9. The method of claim 1 wherein the source of calcium cations is exogenous to the cell.

10. The method of claim 1 wherein the sample contains at least one compound that uncouples oxidative phosphorylation from ATP production.

11. The method of claim 1 wherein the cell comprises at least one polypeptide that is a Bcl-2 family member.

12. The method of claim 1 wherein the cell expresses a gene encoding a polypeptide that regulates cytosolic calcium.

13. The method of claim 12 wherein the gene encodes a mitochondrial calcium uniporter.

14. The method of claim 12 wherein the gene is a transfected gene.

15. The method of claim 14 wherein the gene encodes a mitochondrial calcium uniporter.

16. The method of claim 1 wherein the cell is a permeabilized cell.

17. The method of claim 1 wherein the cell adheres to a solid substrate.

18. The method of claim 1 wherein the cell is a non-adherent cell.

19. (Amended) A method of identifying an agent that uncouples oxidative phosphorylation from ATP production, comprising:

(a) contacting, in each of a plurality of reaction vessels in a high throughput screening array,

(i) a biological sample comprising a cell containing cytosol, a mitochondrion and a calcium indicator molecule, under conditions that permit maintenance of mitochondrial membrane potential, with

(ii) a source of calcium cations,

wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium in the cytosol;

(b) detecting in each reaction vessel the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity;;

(c) repeating steps (a) and (b) at least once; and

(d) comparing (i) the signal generated by the calcium indicator molecule at one or more of said time points prior to and following at least one of the contacting steps in the absence of the candidate agent, to (ii) the signal generated by the calcium indicator molecule at one or more of said time points prior to and following at least one of the contacting steps in the presence of the candidate agent, wherein an increased level of calcium in the cytosol at a time point prior to a contacting step in the presence of the agent, compared to the level of calcium in

the cytosol prior to a contacting step in the absence of the agent, indicates an agent that uncouples oxidative phosphorylation from ATP production.

20. (Amended) A method of identifying an agent that that is a respiratory inhibitor, comprising:

(a) contacting, in each of a plurality of reaction vessels in a high throughput screening array,

(i) a biological sample comprising a cell containing cytosol, a mitochondrion and a calcium indicator molecule, under conditions that permit maintenance of mitochondrial membrane potential, with

(ii) a source of calcium cations,

wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium in the cytosol;

(b) detecting in each reaction vessel the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity;

(c) repeating steps (a) and (b) at least once; and

(d) comparing (i) the signal generated by the calcium indicator molecule at one or more of said time points prior to and following at least one of the contacting steps in the absence of the candidate agent, to (ii) the signal generated by the calcium indicator molecule at one or more of said time points prior to and following at least one of the contacting steps in the presence of the candidate agent, wherein an increased level of calcium in the cytosol at a time point prior to a contacting step in the presence of the agent, compared to the level of calcium in the cytosol prior to a contacting step in the absence of the agent, indicates an agent that is a respiratory inhibitor.

21. (Amended) A method of identifying an agent that alters a mitochondrial calcium uniporter, comprising:

(a) contacting, in each of a plurality of reaction vessels in a high throughput screening array,

(i) a biological sample comprising a cell containing cytosol, a mitochondrion and a calcium indicator molecule, under conditions that permit maintenance of mitochondrial membrane potential, with

(ii) a source of calcium cations,

wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium in the cytosol;

(b) detecting in each reaction vessel the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity;

(c) repeating steps (a) and (b) at least once; and

(d) comparing (i) the signal generated by the calcium indicator molecule at one or more of said time points prior to and following at least one of the contacting steps in the absence of the candidate agent, to (ii) the signal generated by the calcium indicator molecule at one or more of said time points prior to and following at least one of the contacting steps in the presence of the candidate agent, wherein an increased level of calcium in the cytosol at a time point following a contacting step in the presence of the agent, compared to the level of calcium in the cytosol following a contacting step in the absence of the agent, indicates that the agent alters a mitochondrial calcium uniporter.

43. (Amended) A method of identifying an agent that alters mitochondrial function, comprising:

(a) contacting, in each of a plurality of reaction vessels in a high throughput screening array,

(i) a biological sample comprising a cell containing a mitochondrion, cytosol and a calcium indicator molecule, under conditions that permit maintenance of

mitochondrial membrane potential, and wherein the calcium indicator molecule is membrane permeable and capable of generating a detectable signal that is proportional to the level of calcium in the cytosol, with

(ii) a calcium ionophore, under conditions and for a time sufficient to increase calcium levels within the cell;

(b) detecting in each reaction vessel the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and

(c) comparing the signal generated by the calcium indicator molecule at one or more of said time points in the absence of the candidate agent, to the signal generated by the calcium indicator molecule at one or more of said time points in the presence of the candidate agent, and therefrom identifying an agent that alters mitochondrial function.

44. The method of claim 43 wherein the calcium ionophore is selected from the group consisting of ionomycin, A23187, NMDA and a cell depolarization signal.

45. The method of claim 43 wherein the step of contacting is repeated at least once.

46. The method of claim 43 wherein the sample contains at least one compound that alters intracellular distribution of a calcium cation.

47. The method of claim 46 wherein the compound that alters intracellular calcium cation distribution is selected from the group consisting of thapsigargin and Ru360.

48. The method of claim 46 wherein the compound that alters intracellular calcium cation distribution is selected from the group consisting of a calcium ionophore and a membrane permeable compound that alters intracellular calcium distribution.

49. The method of claim 46 wherein the sample contains at least one compound that uncouples oxidative phosphorylation from ATP production.

50. The method of claim 43 wherein the candidate agent is membrane permeable.

51. The method of claim 43 wherein the source of calcium cations is exogenous to the cell.

52. The method of claim 43 wherein the sample contains at least one compound that uncouples oxidative phosphorylation from ATP production.

53. The method of claim 43 wherein the cell comprises at least one polypeptide that is a Bcl-2 family member.

54. The method of claim 43 wherein the cell expresses a gene encoding a polypeptide that regulates cytosolic calcium.

55. The method of claim 54 wherein the gene encodes a mitochondrial calcium uniporter.

56. The method of claim 54 wherein the gene is a transfected gene.

57. The method of claim 56 wherein the gene encodes a mitochondrial calcium uniporter.

58. The method of claim 43 wherein the cell adheres to a solid substrate.

59. The method of claim 43 wherein the cell is a non-adherent cell.

64. (Amended) A method of identifying an agent that alters mitochondrial function, comprising:

(a) contacting, in each of a plurality of reaction vessels in a high throughput screening array,

(i) a biological sample comprising a permeabilized cell depleted of cytosol, a mitochondrion and a calcium indicator molecule, under conditions that permit maintenance of mitochondrial membrane potential, with

(ii) a source of calcium cations,

wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium in the cell;

(b) detecting in each reaction vessel the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and

(c) comparing the signal generated by the calcium indicator molecule at one or more of said time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of said time points in the presence of the candidate agent, and therefrom identifying an agent that alters mitochondrial function.

65. (Amended) The method of ~~either claim 63 or~~ claim 64 wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium in the mitochondrion.

66. (Amended) The method of ~~either claim 63 or~~ claim 64 wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium outside of the mitochondrion.

67. (Amended) The method of ~~either claim 63 or~~ claim 64 wherein the step of contacting is repeated at least once.

68. (Amended) The method of ~~either claim 63 or~~ claim 64 wherein the sample contains at least one compound that alters intracellular distribution of a calcium cation.

69. The method of claim 68 wherein the compound that alters intracellular calcium cation distribution is selected from the group consisting of thapsigargin and Ru360.

70. The method of claim 68 wherein the sample contains at least one compound that uncouples oxidative phosphorylation from ATP production.

71. (Amended) The method of ~~either claim 63 or~~ claim 64 wherein the source of calcium cations is exogenous to the cell.

72. (Amended) The method of ~~either claim 63 or~~ claim 64 wherein the sample contains at least one compound that uncouples oxidative phosphorylation from ATP production.

73. (Amended) The method of ~~either claim 63 or~~ claim 64 wherein the cell comprises at least one polypeptide that is a Bcl-2 family member.

74. (Amended) The method of ~~either claim 63 or~~ claim 64 wherein the cell expresses a gene encoding a polypeptide that regulates cytosolic calcium.

75. The method of claim 74 wherein the gene encodes a mitochondrial calcium uniporter.

76. The method of claim 74 wherein the gene is a transfected gene.

77. The method of claim 76 wherein the gene encodes a mitochondrial calcium uniporter.

78. (Amended) The method of ~~either claim 63 or~~ claim 64 wherein the cell adheres to a solid substrate.

79. (Amended) The method of ~~either claim 63 or~~ claim 64 wherein the cell is a non-adherent cell.

80. (Amended) A method of identifying an agent that alters mitochondrial function, comprising:

(a) contacting, in each of a plurality of reaction vessels in a high throughput screening array,

(i) a biological sample comprising one or more isolated mitochondria and a calcium indicator molecule in a medium, under conditions that permit maintenance of mitochondrial membrane potential, with

(ii) a source of calcium cations,

wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium in the biological sample;

(b) detecting in each reaction vessel the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and

(c) comparing the signal generated by the calcium indicator molecule at one or more of said time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of said time points in the presence of the candidate agent, and therefrom identifying an agent that alters mitochondrial function.

81. (Amended) A method of identifying an agent that alters mitochondrial function, comprising:

(a) contacting

(i) a biological sample comprising one or more isolated mitochondria and a calcium indicator molecule in a medium, under conditions that permit maintenance of mitochondrial membrane potential, with

(ii) a source of calcium cations,

wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium in the biological sample;

(b) detecting the signal generated by the calcium indicator molecule at a plurality of time points, under conditions that permit identification of (i) mitochondrial calcium uniporter activity and (ii) mitochondrial uncoupler or respiratory inhibitor activity; and

(c) comparing the signal generated by the calcium indicator molecule at one or more of said time points in the absence of a candidate agent, to the signal generated by the calcium indicator molecule at one or more of said time points in the presence of the candidate agent, and therefrom identifying an agent that alters mitochondrial function.

82. The method of either claim 80 or claim 81 wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium in the mitochondrion.

83. The method of either claim 80 or claim 81 wherein the calcium indicator molecule is capable of generating a detectable signal that is proportional to the level of calcium outside of the mitochondrion.

84. The method of either claim 80 or claim 81 wherein the step of contacting is repeated at least once.

85. The method of either claim 80 or claim 81 wherein the sample contains at least one compound that alters distribution of a calcium cation in the sample.

86. The method of claim 85 wherein the compound that alters calcium cation distribution is selected from the group consisting of thapsigargin and Ru360.

87. The method of claim 85 wherein the sample contains at least one compound that uncouples oxidative phosphorylation from ATP production.

88. The method of either claim 80 or claim 81 wherein the isolated mitochondria are derived from a cell that comprises at least one polypeptide that is a Bcl-2 family member.

89. The method of either claim 80 or claim 81 wherein the isolated mitochondria are derived from a cell that expresses a gene encoding a polypeptide that regulates cytosolic calcium.

90. The method of claim 89 wherein the gene encodes a mitochondrial calcium uniporter.

91. The method of claim 89 wherein the gene is a transfected gene.

92. The method of claim 91 wherein the gene encodes a mitochondrial calcium uniporter.

93. (Amended) The method of any one of claims 1, 21, ~~22, 42, 63,~~ 64, 80 or ~~89~~ 81 wherein subsequent to the step of contacting the biological sample with the source of calcium cations and prior to the step of comparing signals, the biological sample is contacted (i) with at least one compound that uncouples oxidative phosphorylation from ATP production, and (ii) with at least one agent that alters mitochondrial function.

94. The method of claim 93 wherein the agent that alters mitochondrial function is cyclosporin A.

95. The method of claim 93 wherein the agent that alters mitochondrial function is selected from the group consisting of cyclosporin A, rotenone, oligomycin, succinate and Bcl-2.

96. The method of claim 93 wherein the compound that uncouples oxidative phosphorylation from ATP production is selected from the group consisting of FCCP and CCCP.

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